

Interaction of reduced lysozyme with surfactants.

Disulfide effects on reformed structure in micelles

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Abstract

The reformation of secondary structure for unfolded, disulfide reduced hen egg white lysozyme (HEWL) upon interaction with surfactants was studied using CD, fluorescence and IR (infrared) techniques. Equilibrium CD studies showed that reduced HEWL when mixed with negatively charged surfactants, such as SDS (sodium dodecyl sulfate), gradually regains average helical structure to a level equivalent to that obtained for the oxidized form also in SDS, but both forms lose tertiary structure in such environments. This non-native structure recovery process begins with monomer surfactant interaction but at higher concentrations is in part dependent on micelle formation, with the helical fraction reaching its maximum value with each surfactant only above the CMC. Fluorescence changes were more complex, evidencing an intermediate state at lower surfactant concentration. With positively charged surfactants the degree of helicity recovered was less, and the intermediate state in fluorescence was not seen. Stopped flow dynamics studies showed the CD kinetics fit to two exponentials as did the fluorescence. The faster steps in CD and fluorescence detected kinetics appear to be correlated which suggests formation of an intermediate on rapid interaction of the micelle and protein. The second step then reflected attainment of a stable surfactant solvated state which attains maximum helicity and moves the Trps to a more hydrophobic environment, which may occur in independent steps, as the slower kinetics are not well correlated.

Keywords: disulfide bond, reduction, surfactant, micelle, secondary structure, membrane

Introduction

Protein folding remains a vital area of biophysical research. Many proteins fold spontaneously in a manner that seems to be based solely on their sequence, while others misfold or require external conditions or chaperones to aid in folding. Sequence dependence of folding has dominated many studies over the past few decades, and remains a fundamental issue. One special aspect arising from the sequence is the role of Cys and the presence or absence of disulfide bonds in the folding sequence. Forming the correct disulfide bonds before the polymer folds to a stable secondary and tertiary structure would greatly limit the conformational entropy, and thus facilitate the search for a global minimum structure. Conversely forming the wrong disulfide bond will insert a misfolding step into the mechanism and dramatically slow progress toward the minimum energy structure.

Previous studies have shown that reduction of disulfide bonds decreases the stability of the native protein [1-5]. Some studies have maintained that disulfides have a stabilizing effect on the native state but do not determine either the folding pathway or the final three-dimensional structure of the protein, so that in such cases disulfide links can be formed at late stages of the folding process [6]. Other studies contradict this point of view and show that native disulfide bonds can greatly accelerate secondary structure formation at early stages of the folding process when the protein has no defined tertiary structure [7]. The effects of disulfide bond formation in the folding process remain unclear: does correct disulfide bond formation determine the folding pathway or just stabilize the elements of secondary structures formed and contribute to the formation of tertiary structure? Sorting these out for proteins in only buffer conditions is challenging because many reduced proteins do not fold, so no sensible comparison of structure or stability is possible with the oxidized form.

Ionic surfactants can interact strongly with oppositely charged globular proteins, often resulting in denaturation [8]. Such studies have been long pursued; however, the mechanism by which the surfactants influence protein structure is still not well defined. Bovine serum albumin (BSA) has been most frequently studied with sodium dodecyl sulfate (SDS), a representative anionic surfactant [9-12]. Even millimolar levels of SDS are known to be a strong denaturant for many proteins [10, 13], yet SDS can also counteract the effect of other denaturants [14, 15]. We have shown it to lead to compact, highly helical (molten globular) structures for acid denatured cytochrome c [16, 17] and to induce sheet-to-helix transformations in β -lactoglobulin [18-20]. These effects can be strongly concentration sensitive, with the latter results being seen for proteins in micellar environments. Such surfactants may provide a simple model for study of protein-membrane like interactions (since both offer an interfacial interaction where charge and hydrophobic elements are separated or organized at some level). These can have some advantages for biophysical studies due to higher solubility and smaller size of surfactants as micellar forms. Membrane interaction (and by extension, surfactants) can also offer an alternative folding pathway which may illustrate the relative role of disulfide bond formation in a consistent medium. Such environments do allow both reduced and oxidized forms to fold to some structure which can then be compared and may or may not be related to the native state structure. Kinetic studies of SDS interaction with proteins are less common than are equilibrium evaluations, but several studies have been undertaken monitoring heme absorbance changes and near uv CD (circular dichroism) [21], fluorescence changes [22], and include our previous CD and fluorescence study of acid denatured Cyt c [16].

In this study we focus on hen egg white lysozyme (HEWL), which has four disulfide bonds in its native state (indicated schematically in Figure S1, supplement), and compare effects

on folding, or regaining structure to a new state, by study of the reduced form of the protein as induced by interaction with various surfactants which provide a model system for study of charge and hydrophobic interactions. These systems may mimic protein-membrane interactions, which we will report separately (Zhu and Keiderling, to be submitted), although the structures and presumably the detailed interaction mechanisms are admittedly different. It is interesting to note that, for some Gram-negative bacteria, native lysozyme or partially unfolded forms of lysozyme can penetrate the outer membrane and acquire bactericidal capacity [23-25]. The mechanism of the antimicrobial properties of lysozyme on Gram-negative bacteria is still unclear, and the driving forces behind such interactions of membrane surfaces and the HEWL structure under such conditions remain an interesting problem.

Experimental

Materials. Lysozyme from hen egg white (HEWL) and lyophilized *Micrococcus lysodeikticus* cell were purchased from Sigma. A comparative test experiment was carried out on a mutant lysozyme in which all the cysteines are replaced by alanines which was kindly provided by Prof. Harald Schwalbe, Frankfurt University, and will be reported separately. The surfactants used (compared in Fig. S2, supplement): sodium dodecyl sulfate (SDS), sodium 1-dodecanesulfonate ($C_{12}SO_3$), sodium 1-decanesulfonate ($C_{10}SO_3$) and sulfobetaine (SB3-10) were purchased from Sigma except for dodecyltrimethylammonium chloride (DTAC) which was from Fluka. The reducing agent 1,4-dithio-DL-threitol (DTT) was purchased from Fluka. Solution pH was measured with a HANNA HI 98180 pH meter. All the materials were used without further purification. To prepare reduced lysozyme solution, the protein was dissolved (0.4 mg/mL) in 20 mM pH 4.6 phosphate buffer and incubated with 5 mM DTT for 24 hours at 65 °C. The

reduction of the sample was quenched by rapid cooling and then storage at 5 °C. This procedure is a modification of previously published methods [26]. All samples were studied at pH 4.6 to enhance solubility of the reduced form with DTT reductant. CMC (critical micelle concentration) for the various surfactants under the experimental conditions, which include buffer, lowered pH and reductant, were determined by absorbance of BZA (benzoylacetone or 1-phenyl-1,3-butadione) using literature methods [27].

CD measurements. CD spectra were measured from 185 nm to 250 nm with a 50 nm/min scanning rate, 2 s response time, 1 nm bandwidth and as the average of 8 scans on a JASCO 810 spectrometer (Jasco, Inc.). For equilibrium measurements, the protein solutions were prepared at 0.2 mg/mL in 20 mM pH 4.6 phosphate buffer and measured in a 1 mm pathlength quartz cuvette (Starna, Inc) at room temperature. All sample spectra were corrected by subtraction of the corresponding spectrum of the buffer. The fractional secondary structures were calculated with the CDPro programs (accessible at: lamar.colostate.edu/~sreeram/CDPro/main.html) using data for 195 nm to 250 nm [28].

Fluorescence. Fluorescence spectra were measured on a Fluoromax-3 spectrofluorometer (JobinYvon Inc.) with the samples in a 1 mm×1 cm quartz cell. The excitation wavelength was 295 nm and the emission spectra were collected from 300 nm to 500 nm. All the spectra were corrected by subtraction of the buffer spectrum.

Stopped-flow measurements. For stopped-flow mixing dynamics, an SFM-400 accessory (Biologic) and MPS-52 control console were connected to the Jasco-810 spectrometer. The Xe lamp in the CD spectrometer was changed to a Xe-Hg lamp to enhance far-UV intensity. The mixing deadtime under our measurement conditions was ~10 ms, but instrument response was much faster and the signal change large enough to allow determination of kinetics in the ms and longer

time range. The CD signal channel was recorded with 1 KHz sampling for 5 s of the signal at 222 nm using a 4 nm bandwidth by means of standard JASCO kinetics methods with a fixed HV and normalization to the detector current (in the software) to determine ΔA with a faster response. The total fluorescence signal was simultaneously collected on the same sample for all emission above 320 nm using a cut-off filter in front of a separate fluorescence detector which was located at right angles to the CD and excitation beam. The results were fitted with multiple exponential functions using the Bio-Kin32 software.

Lysozyme activity assay. The enzymatic activity of lysozyme was measured by monitoring the rate of decrease in the turbidity of a *Micrococcuslysodeikticus* cell suspension. For this 0.02 ml of lysozyme sample (0.1 mg/ml) was added to a 1 cm cuvette with 0.98 ml cell suspension (0.3 mg/ml), which was then was agitated via a pipette action for 10 s. The absorbance was recorded at 450 nm on a CARY 300 Bio UV/Vis (Varian) spectrometer every 10 s for 10 minutes. One unit of activity corresponds to an absorbance decrease of 0.0026/min.

Results and Discussion

The refolding of reduced HEWL with anionic surfactant micelles. After reduction HEWL loses all four S-S bonds as demonstrated by mass spectra which show the molecular weight to increase from the native state value by eight mass units (data not shown). CD spectra (Fig. 1, truncated at 200 nm because spectra became prohibitively noisy at <200 nm due to absorbance of the reductant and SDS) show that the reduced/unfolded HEWL (blue dotted trace) has a higher fraction of unordered structure and much less helical structure than the native state (red dash line). The fluorescence spectra (Fig. 2) of the reduced HEWL (red solid line) has 50% more intensity than that of the native state (black solid line), and a red shift of the peak position from

344 to 354 nm is observed which is consistent with the tryptophans being more exposed to solvent after reduction. (The lower pH of 4.6 is needed for solubility of the protein in reductant, but has little effect on native HEWL.) Low concentrations of negatively charged surfactants (e.g. SDS) cause HEWL to aggregate and precipitate, limiting the range of concentrations we can study HEWL-surfactant interaction. Reduced HEWL is somewhat more soluble in SDS, but concentrations below 1-2 mM remain problematic.

The far-UV CD spectra of reduced HEWL in the presence and in the absence of anionic surfactant SDS as compared to that of native HEWL are shown in Fig. 1 (a), and the average fractional secondary structure derived from these spectra (using CDPro) are shown in Fig. 1 (b). The CD spectra show that with the addition of anionic surfactant, SDS, the reduced HEWL structure becomes increasingly helical, coming to a saturated value well above the critical micelle concentration (CMC) in this environment (2.7 mM as corrected for buffer, reduced pH, protein and reductant). Reduced HEWL with SDS above the CMC has even more helical structure than does the native state. However, adding SDS to native HEWL also increases its helicity (Fig. 3), so that both reduced and oxidized HEWL in SDS above its CMC have similar fractional helical compositions. [While native HEWL has ~32% helix, it increases to ~37% for the oxidized form in micellar SDS. The CMC is buffer and pH sensitive, such that the altered conditions of our experiment yield lower values than often quoted in the literature, as we have determined by absorbance change of BZA (benzoylacetone or 1-phenyl-1,3-butadione). The relevant values for our experiments are indicated on the figures (and summarized in Table S1, Supplement). These plots show that the maximal gain of helical structure occurs well above the CMC, indicating monomer surfactant-protein interaction is insufficient for saturation and that micelles have a role in the final state.]

The intrinsic fluorescence emission from the six tryptophan residues undergoes a two-step change upon interaction with SDS. The fluorescence intensity first increased at low SDS concentration and then decreased back to the level of native HEWL upon further addition of SDS to higher concentration (10 mM, Figure 2b). The fluorescence peak position for reduced HEWL in SDS also changed in a similar manner: first becoming blue shifted and then red shifted back to nearly the native (oxidized) HEWL value (342 nm). At low concentrations of SDS, surfactant monomers with negative charges can bind specifically to the positively charged sites and neutralize the protein, which leads to some helical formation and considerable increase in fluorescence intensity and blue shift [29]. Increasing the SDS concentration increased hydrophobic interaction between the reduced HEWL and SDS, which steadily increases the initially reduced HEWL fluorescence until the CMC is reached, whereupon the hydrophobic interaction with the protein appears to become limited by micelle formation. Above the CMC the helicity continues to grow but the fluorescence characteristics reverse, quenching intensity and red-shifting the maximum frequency, indicating an altered interaction with micelles having increased exposure to solvent and charge. For the micellar saturated reduced HEWL state, which has similar average secondary structure content to the oxidized micellar state (i.e. for oxidized HEWL with SDS above CMC). The reduced HEWL in SDS fluorescence also has similar frequency to the native state with and without SDS (see open red circles, Fig. 2b). (None-the-less, despite the fluorescence recovery, both oxidized and reduced HEWL have non-native tertiary structure in SDS micelles, see below.)

Near-UV CD can monitor changes in Trp environments and by extension changes in tertiary structure. In Fig. 4 are compared the near-UV CD for native and reduced HEWL with and without SDS addition. The spectra vary and the CD intensity at 290 nm which indicates Trp

structure for oxidized HEWL is lost in the SDS interacting species. Reduced HEWL tertiary structure does change with addition of SDS, but the native form is not recovered. By comparison, the form of the near-UV CD for oxidized HEWL in SDS is also different, indicating a loss of tertiary structure in both forms that is not recovered in the surfactant medium.

The balance of the electrostatic interactions and hydrophobic interactions between protein and surfactants are important aspects in this process of reforming helical structure. Two more surfactants were studied that alter the relative distribution of negative charge and hydrophobicity. $C_{12}SO_3$ has the same length hydrophobic tail as SDS but contains a different head group with less partial negative charge distribution [30]. Interaction with reduced HEWL results in the same effects on the spectra, and by extension on the structure, as found with SDS, but requires a higher concentration of $C_{12}SO_3$ to restore secondary structure for the reduced HEWL (Fig. S3, Supplement). As seen with SDS, more helical structure can be obtained for reduced HEWL under conditions above the $C_{12}SO_3$ CMC than for native state HEWL. Paralleling the SDS result, the fluorescence intensity first increased and then decreased back to a level characteristic of native HEWL upon addition of $C_{12}SO_3$, and the fluorescence peak position first blue shifted and then red shifted back to the native HEWL position. By contrast $C_{10}SO_3$ has the same head group, and thus charge, as $C_{12}SO_3$ but has a shorter hydrophobic tail and higher CMC, and thus paralleling the overall CMC dependent pattern, it requires even higher concentration to induce full recovery of the helical fraction in reduced HEWL (Fig. S4, Supplement). This contrast in behavior for different length surfactants reinforces the importance of the micellar formation to induce the maximum helicity for each surfactant with this protein. In the same way as seen with SDS, both $C_{12}SO_3$ and $C_{10}SO_3$ can restore helical content for reduced HEWL when the surfactants interact with the protein above their CMC, but these two $-SO_3$ based surfactants

require higher concentrations, which appears to be due to their having less charge on their head groups and, for $C_{10}SO_3$, less hydrophobic interaction. The difference in hydrophobicity between $C_{10}SO_3$ and $C_{12}SO_3$ alters the CMC and that may be the critical parameter for achieving ultimate structural change in reduced HEWL. These comparisons lead to the added conclusion that more negative charge and more hydrophobicity on the anionic surfactant will favor the process of regaining secondary structure and altering Trp exposure in reduced HEWL.

The interaction of reduced HEWL with cationic and zwitterionic surfactant micelles. While anionic surfactant micelles should have specific binding to the positively charged reduced HEWL protein at these reduced pH 4.6 values (since the HEWL isoelectric point is 10.7), for cationic surfactant micelles we might expect different behavior, even though both offer hydrophobic interactions. In contrast to its behavior in anionic surfactants, the reduced HEWL can only partially recover helical structure by addition of the cationic surfactant, DTAC, even for concentrations above the DTAC CMC (Fig. 5). The helical fraction for reduced HEWL in DTAC above the CMC is only ~25% which remains below the ~33% helix in native HEWL structures. Fig. 5 (b) shows the fluorescence intensity and peak position change upon adding DTAC, which is a simpler change than with the anionic surfactants, showing only further intensity increase and blue shift from native HEWL, both aspects stabilizing above the CMC. For the zwitterionic surfactant, Sulfobetaine (SB3-10), similar effects on the structures of reduced HEWL were seen as for DTAC, implying that the hydrophobic interaction is primarily operative for both of these with HEWL (see Fig. S5, Supplement).

Comparison of the effects of different surfactants on the refolding of reduced HEWL suggests that negative charge on surfactants is required to specifically bind to the positively charged protein and to refold and increase the helical fraction of secondary structure for the reduced

HEWL to an extent comparable to or above that of the native protein. Native HEWL also increases helicity and loses tertiary structure when interacting with negative surfactant micelles, showing a parallel behavior with and without disulfides that is not obvious in only buffer environments. However, with positive micelles, DTAC, there is little change in secondary structure (Fig. 3a), although the fluorescence changes (Fig.3b) indicate an interaction with surface Trp residues. Analogous experiments with a mutant HEWL where all cysteines were converted to alanines gave similar variations with SDS and DTAC (unpublished results) further supporting the role of disulfides in this difference.

Refolding of helical structure in reduced HEWL induced with trifluoroethanol (TFE). TFE is known to induce helical structures in proteins, not only by disturbing native hydrophobic interactions, binding to the protein backbone via hydrogen bonds, but also providing hydrophobic surface areas to mimic interaction of the polypeptide chain with membranes. The CD spectra (Fig. S6 in supplement) show that increasing TFE concentration can steadily induce formation of more helical structure in HEWL. At 50% TFE, both reduced and oxidized HEWL have increased ellipticity, more than twice that of native HEWL in aqueous buffer. At low concentration of TFE (15%) there is no significant effect on the oxidized HEWL secondary structure, but reduced HEWL gains helical structure at even lower concentrations of TFE. This may be due to the reduced HEWL not being as compact as native HEWL resulting in its hydrophobic sites being more exposed to the solvent, and thus the TFE having more relative impact on structure. However, the conformational transition is virtually complete at 50% TFE for both reduced HEWL and oxidized HEWL suggesting that the binding of TFE to surface sites is saturated, forming a direct parallel to the above (negative) surfactant results. It is interesting that with stepwise addition of TFE, reduced HEWL does not pass through an intermediate state that

matches the secondary structure of the oxidized state HEWL at some specific concentration of TFE. Presumably this is because the original hydrophobic interactions of the protein are disrupted once TFE is added, and the refolding is taking place on a different path to another, partially helical but non-native structure.

With increasing TFE concentration, the fluorescence intensity first increases then decreases back and the peak position blue-shifts then red-shifts back, much as seen with the anionic surfactants (Fig. S6, Supplement). At low concentration, TFE binds to sites on the protein to form hydrogen bonds and make the tryptophans less exposed to solvent [31-34]. When TFE binds to more hydrophobic surface sites at higher concentrations and disrupts hydrophobic interactions between helical segments, the tryptophans will most likely be more solvent exposed.

Thermal stability studies on the refolded state of reduced HEWL in surfactant micelles.

The thermal stability studies shown in Fig. 6 show a nearly linear decrease in the (negative) ellipticity magnitude in the far-UV CD on heating the highly helical state of reduced HEWL in surfactant micelles. This can be contrasted with the clearly cooperative thermal denaturation of native HEWL (filled squares, Fig. 6), which means the cooperativity of structural stabilization is lost for the helical states of reduced HEWL in surfactant micelles. This would be consistent with formation of a conformation, often termed a molten globule, in the presence of micelles that has substantial secondary structure (helix) but little tertiary structure. This may represent a dynamic equilibrium of related conformational substates resulting in the loss in cooperativity in the structural transition. It is important to see that addition of anionic or cationic surfactant to native HEWL also resulted in a loss of cooperative unfolding (Fig. 6), indicating the surfactant had a similar effect on oxidized state tertiary structure. In other words, inclusion of disulfides does not change the surfactant effect on cooperativity.

Stopped-flow kinetic study of the refolding of reduced HEWL with surfactant micelles.

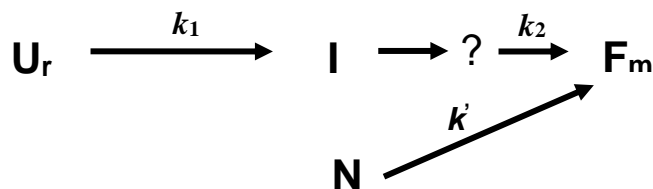
The kinetics of the structural changes induced in reduced HEWL by interaction with surfactant micelles were monitored by measuring both CD ellipticity change at 222 nm and total fluorescence intensity above 320 nm under stopped-flow mixing conditions. The and fluorescence kinetic traces for reduced HEWL refolding with anionic (SDS) and cationic (DTAC) surfactant micelles are shown in Fig. 7 and Fig. 8, respectively CD (these figures show fits only for CD, see Fig. S9, Supplement, for example of raw data). The stopped-flow curves, $\phi(t)$, are fit with a multiple exponential function to determine rate constants in the structure reforming process using the following form:

$$\phi(t) = at + b + \sum c_i \cdot e^{-k_i t} \quad (\text{eqn. 1})$$

where k_i and c_i are the rate constant and amplitude of the i th phase and a and b represent linear baseline corrections.

The stopped-flow kinetics of the reduced HEWL structure change with SDS micelles show a biexponential behavior for the ellipticity change at 222 nm. The rate constants obtained from the stopped-flow data analysis at various concentrations are listed in Table I. The faster CD determined rate constant, $k_1 \sim 250 \text{ s}^{-1}$, corresponds to a fast phase which results in a large gain in helical secondary structure. This fast phase is followed by a slower kinetic phase with a rate constant, $k_2 \sim 10 \text{ s}^{-1}$, which indicates relatively small structural change after further interaction with SDS micelles. The fluorescence curves also show an initial fast decrease with a rate constant, $k_1 \sim 150 \text{ s}^{-1}$, which is followed by a slow decrease with a rate constant, $k_2 \sim 0.7\text{-}1.8 \text{ s}^{-1}$. This indicates that there is a collapse of the polypeptide chain accompanying the major secondary structure change at the very early stages of folding in the fast phase, in other words, these fast CD and fluorescence spectral changes may result from the same process or kinetic

step. The rate constants are similar with variation in concentration above the CMC, with a modest tendency for the fast CD constant to decrease and the fast fluorescence constant to increase with increase in SDS micelle concentration. This opposite trend suggests CD and fluorescence may measure different overlapping processes. It is important to note that the CD kinetic data is quite noisy with fast steps that are close to our mixing time limits, so there may be additional, undetected fast steps or added (systematic) error in the fast step kinetic constant determinations, which would make discrimination between CD and fluorescence results and these concentration trends tentative. However the slower rate constants (k_2) appear to correspond to different processes which may result from one or multiple sequential mechanistic steps. We propose that these steps correspond to a qualitative kinetic mechanism for the refolding of reduced/unfolded HEWL with surfactant micelles as follows:



Scheme 1. Proposed mechanism for refolding of reduced HEWL, showing the possibility of multiple intermediates.

In scheme I, \mathbf{U}_r represents the unfolded state after reduction; \mathbf{N} denotes the native state; \mathbf{I} is an intermediate state and \mathbf{F}_m is the highly helical, compact state for HEWL in surfactant micelles. \mathbf{I} is formed in the fast kinetic phase corresponding to refolding of much of the secondary structure. The \mathbf{I} state will further fold to the \mathbf{F}_m state, possibly through added intermediate steps, which has even more helical structure than the native state (\mathbf{N}), in the second kinetic phase as induced by

further interaction with SDS micelles. As noted above, this latter process may be multistate, given the differences in the CD and fluorescence k_2 values.

The state of reduced HEWL in SDS micelles (F_m) has almost the same fractional secondary structure and similar tertiary structure as does oxidized HEWL in SDS micelles, which is supported by CD (both near and far-UV) and fluorescence measurements. We also measured the unfolding kinetics of native state HEWL to the F_m state, and with CD detection a single exponential was determined yielding a rate constant, $k' \sim 10 \text{ s}^{-1}$, which is close to the CD determined k_2 for the reduced HEWL interacting with micelles. The fluorescence change was again biexponential for HEWL in SDS, but with slower rate constant than for the reduced case. The interaction between the native state N and SDS micelles thus appears to undergo a kinetic phase with a rapid change of Trp environment (presumably due to the charge interaction) followed by a slower structure change with a rate constant $\sim 10 \text{ s}^{-1}$ that encompasses formation of more helical structure as well as loss of tertiary structure. The latter step may be similar to that of the intermediate state, I, in the reduced HEWL restructuring (Scheme 1) both resulting in molten globule like states, F_m , of high helicity..

The refolding kinetics of reduced HEWL in cationic micelles DTAC (Fig. 8) also show biexponential behavior as is seen with SDS, but the amplitude of the CD ellipticity change at 222 nm is smaller, which is consistent with equilibrium result that the helical fraction for reduced HEWL can not be as fully recovered in cationic as in anionic surfactant micelles. The fluorescence intensity increases in a biexponential change on mixing with DTAC, instead of decreasing as was seen in anionic surfactant micelles, such as SDS.

Comparision with refolding of denatured/reduced HEWL by dilution and reformation of disulfide bonds. Previous papers have reported both equilibrium and kinetic studies on the

refolding of denatured/reduced HEWL, under different conditions than we have studied. Native HEWL can be denatured by GdnHCl or urea and reduced by addition of DTT [35-37]. The refolding process is accomplished by dilution of the denatured/reduced HEWL with added refolding buffer containing GSSG which can oxidize the Cys and allow reformation of the protein disulfide bonds. The idea behind those previous studies is to remove the effect of the denaturant and reform the disulfide bond. Our experiment is unique and different from these reported studies because we do not reoxidize and reform the disulfide but instead have added micelles which can provide an alternative pathway for structure formation that can then be compared to that including disulfide bonds in the same environment. These previous refolding studies reported three rate constants with k_1 and k_2 close to 0.06 min^{-1} and k_3 close to 0.002 min^{-1} which are all much slower than the rate constants we have obtained with surfactants, $k_1 \sim 250 \text{ s}^{-1}$ and $k_2 \sim 10 \text{ s}^{-1}$. The explanation for this difference is because it takes time for the Cys to locate each other, form disulfide bonds and then reshuffle to the correct configuration of -S-S- links. Our experiment does not require formation of disulfide bonds, so the kinetics are not limited by that rate, but by contrast monitors the secondary structure refolding mechanism, which is occurring on a much faster time scale. Chaffotte et al. also studied refolding of oxidized HEWL which has already formed all disulfide bonds by dilution with refolding buffer and reported two rate constants with the fast rate constant $k_1 = 69.9 \text{ s}^{-1}$ and the slower rate constant $k_2 = 2.74 \text{ s}^{-1}$ [36]. These reported values are comparable with our kinetic results, but imply that micelles can provide faster secondary structure formation mechanisms than disulfide bonds, even when disulfide bonds are already present.

Conclusion

Interaction of reduced HEWL in surfactant micelles. Reduced HEWL interacts with anionic surfactants above their CMC to regain a highly helical but non-native state. This non-native state has more helical structure than that of the native HEWL but loses tertiary structure and enzymatic activity. In our studies, this non-native helical state of the reduced protein can not be distinguished from that of oxidized (originally native state) HEWL once it interacts with micelles since they both have superimposable CD and fluorescence spectra and no enzymatic activity. Charge and hydrophobicity play important roles in this non-native refolding process, particularly since we are studying HEWL, which has a pI~10-11, at pH 4.6. More negative charge on the surfactant head groups helps micelles bind to the positive protein surface (of HEWL), effectively desolvating it. This is operative at low surfactant concentrations involving monomer interactions inducing initial structure change. Larger hydrophobicity, where the differences are most evident with micelle formation, can induce even more helical structure (i.e. favoring internal H-bond formation leading to stability). Reduced HEWL can only gain part of this degree of helicity when interacting with cationic surfactants above their CMC, since the surfactant has the same charge as the protein, leading to binding that effectively only depends on the hydrophobic aspects. Our data suggest that the surfactant acts primarily to desolvate the peptide chain, which can allow those segments having a high helical propensity to reform structure. As such it suggests that the hydrophobic interactions are not sufficient to drive the protein to such a fully structured state, and that the framework needs an assist to build secondary structure, native or otherwise. This desolvation is supported by the parallel between surfactant and TFE influences on regaining helicity, however their fluorescence differences suggest that the terminal (tertiary at least) states are not the same. The preference for negative (anionic) surfactants suggests that neutralization of positive sidegroups can facilitate structure formation, which may be akin to creating hydrophobic

interactions, or reducing charge repulsion, thereby reducing barriers to folding found in the just buffer-solvated, positively charged protein structure. Thus, although average secondary structure is similar in the two proteins interacting with SDS, native (oxidized) and reduced, it is possible and even likely that their detailed (especially tertiary) structures differ.

The role of disulfide bonds on the folding pathway of HEWL. From both equilibrium and kinetic studies of the reformation of secondary structure for reduced HEWL in surfactant micelles, we have shown that the reduced HEWL state, U_r , without disulfide bonds can be reconfigured to a compact state of higher helicity state, F_m , in micelles. This also behaves like the partially (tertiary structure) unfolded, but more helical state of native (oxidized) HEWL interacting with anionic surfactant micelles, where the micelles provide counteracting destabilizing (tertiary) and stabilizing (helicity) forces such as electrostatic and hydrophobic interactions, respectively. Both native and reduced HEWL in micelles have structural characteristics often associated with molten globules.

The formation of disulfide bonds early in protein folding clearly provide a restriction of conformational space, which can facilitate folding in, for example, a funnel mechanism. However, such disulfide links are not required to allow the reduced protein to find a compact state that has considerable secondary structure, although the tertiary structure may differ from that of the native (oxidized) state, as it does here for HEWL. This parallel of oxidized and reduced HEWL folding, when in surfactant micellar media, as found in both our equilibrium and dynamic studies using CD and fluorescence, suggests that disulfide bonds do not determine either the folding pathway or the final secondary structure of the protein at early stages of the folding process. Rather it suggests that disulfides can add stability to elements of secondary structures formed and contribute to the formation of tertiary structure.

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References:

- [1] K. Ryota, K. Inaka, Y. Taniyama, S. Kidokoro, M. Matsushima, M. Kikuchi and K. Yutani, Enthalpic destabilization of a mutant human lysozyme lacking a disulfide bridge between cysteine-77 and cysteine-95, *Biochemistry* 31 (1992) 8323-8328.
- [2] S.E. Radford, D.N. Woolfson, S.R. Martin, G. Lowe and C.M. Dobson, A three-disulphide derivative of hen lysozyme. Structure, dynamics and stability., *Biochem J.* 273 (1991) 211-217.
- [3] P.L. Privalov, E.I. Tiktopulo, S.Y. Venyaminov, Y.V. Griko, G.I. Makhatadze and N.N. Khechinashvili, Heat capacity and conformation of proteins in the denatured state *J. Mol. Biol.* 205 (1989) 737-750.
- [4] T.E. Creighton, Disulphide bonds and protein stability, *BioEssays* 8 (1988) 57-63.
- [5] T.E. Creighton, Interactions between cysteine residues as probes of protein conformation: The disulphide bond between Cys-14 and Cys-38 of the pancreatic trypsin inhibitor, *J. Mol. Biol.* 96 (1975) 767-776.
- [6] R. Jaenicke and J. Buchner, Protein folding: From “unboiling an egg” to “catalysis of folding.” *Chemtracts-Biochem. & Mol. Biol.* 4 (1993) 1-30.
- [7] M.E. Goldberg and Y. Guillou, Native disulfide bonds greatly accelerate secondary structure formation in the folding of lysozyme, *Protein Sci.* 3 (1994) 883-887.
- [8] M.N. Jones and P. Manley, Interaction between lysozyme and n-alkyl sulphates in aqueous solution., *J. Chem. Soc., Faraday Trans. 1* 76 (1980) 654–664.
- [9] E.L. Gelamo, C.H.T.P. Silva, H. Imasato and M. Tabak, Interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants: spectroscopy and modelling, *Biochim. Biophys. Acta* 1594 (2002) 84-99.
- [10] E.L. Gelamo and M. Tabak, Spectroscopic studies on the interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants, *Spectrochim. Acta A* 56 (2000) 2255-2271.
- [11] S. Lapanje, Physicochemical aspects of protein denaturation., Wiley Interscience Publications, New York 1978.
- [12] M.N. Jones, Biological interfaces: An introduction to the surface and colloid science of biochemical and biological system, Elsevier Scientific Pub. Co., Amsterdam, 1975.
- [13] N.J. Turro, X.-G. Lei, K.P. Ananthapadmanabhan and M. Aronson, Spectroscopic probe analysis of protein-surfactant Interactions: The BSA/SDS system, *Langmuir* 11 (1995) 2525–2533.

- [14] Y. Moriyama and K. Takeda, Re-formation of the helical structure of human serum albumin by the addition of small amounts of sodium dodecyl sulfate after the disruption of the structure by urea. A comparison with bovine serum albumin, *Langmuir* 15 (1999) 2003–2008.
- [15] E.L. Duggan and J.M. Luck, The combination of organic anions with serum albumin: IV. Stabilization against urea denaturation, *J. Biol. Chem.* 172 (1948) 205-220.
- [16] Q. Xu and T.A. Keiderling, Stop-flow kinetics studies of the interaction of surfactant, sodium dodecyl sulfate, with acid-denatured cytochrome c, *Proteins: Struct., Funct., Bioinf.* 63 (2006) 571–580.
- [17] Q. Xu and T.A. Keiderling, Effect of sodium dodecyl sulfate on folding and thermal stability of acid-denatured cytochrome c: A spectroscopic approach, *Protein Sci.* 13 (2004) 2949–2959.
- [18] N. Ge, X. Zhang and T.A. Keiderling, Kinetic studies of the interaction of β -Lactoglobulin with model membranes: Stopped-flow CD and fluorescence studies, *Biochemistry* 49 (2010) 8831-8838.
- [19] X. Zhang and T.A. Keiderling, Lipid-Induced Conformational Transitions of β -Lactoglobulin, *Biochemistry* 45 (2006) 8444-8452.
- [20] L.K. Creamer, Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β -lactoglobulin, *Biochemistry* 34 (1995) 7170-7176.
- [21] K. Takeda, K. Takahashi and P.P. Batra, Kinetic aspects of the interaction of horse heart cytochrome c with sodium dodecyl sulfate, *Arch. Biochem. Biophys.* 236 (1985) 411-417.
- [22] T.K. Das, S. Mazumdar and S. Mitra, Characterization of a partially unfolded structure of cytochrome c induced by sodium dodecyl sulphate and the kinetics of its refolding., *Eur. J. Biochem.* 254 (1998) 662–670.
- [23] P. Wild, A. Gabrieli, E.M. Schraner, A. Pellegrini, U. Thomas, P.M. Frederik, M.C.A. Stuart and R.V. Fellenberg, Reevaluation of the effect of lysozyme on *Escherichia coli* employing ultrarapid freezing followed by cryoelectronmicroscopy or freeze substitution, *Microsc. Res. Tech.* 39 (1997) 297–304.
- [24] H.R. Ibrahim, S. Higashiguchi, M. Koketsu, L.R. Juneja, M. Kim, T. Yamamoto, Y. Sugimoto and T. Aoki, Partially unfolded lysozyme at neutral pH agglutinates and kills gram-negative and gram-positive bacteria through membrane damage mechanism, *J. Agric. Food Chem.* 44 (1996) 3799–3806.
- [25] H.R. Ibrahim, S. Higashiguchi, L.R. Juneja, M. Kim and T. Yamamoto, A structural phase of heat-denatured lysozyme with novel antimicrobial action, *J. Agric. Food Chem.* 44 (1996) 1416–1423.
- [26] S.S. Ristow and D.B. Wetlaufer, Evidence for nucleation in the folding of reduced hen egg lysozyme, *Biochem. Biophys. Res. Commun.* 50 (1973) 544-550.
- [27] A. Domínguez, A. Fernández, N. González, E. Iglesias and L. Montenegro, Determination of Critical Micelle Concentration of Some Surfactants by Three Techniques, *J. Chem. Educ.* 74 (1997) 1227-1231.
- [28] N. Sreerama and R.W. Woody, Computation and analysis of protein circular dichroism spectra, *Methods Enzymol* 383 (2004) 318-351.
- [29] M.D. Lad, V.M. Ledger, B. Briggs, R.J. Green and R.A. Frazier, Analysis of the SDS-lysozyme binding isotherm, *Langmuir* 19 (2003) 5098-5103.
- [30] P.D.T. Huibers, Quantum-chemical calculations of the charge distribution in ionic surfactants, *Langmuir* 15 (1999) 7546–7550.

- [31] F. Rashid, S. Sharma, M.A. Baig and B. Bano, Molten globule state of human placental cystatin (HPC) at low pH conditions and the effects of trifluoroethanol (TFE) and methanol, *Biochem. Cell Biol.* 84 (2006) 126-134.
- [32] K. Gast, D. Zirwer, M. Müller-Frohne and G. Damaschun, Trifluoroethanol-induced conformational transitions of proteins: Insights gained from the differences between α -lactalbumin and ribonuclease A, *Protein Sci.* 8 (1999) 625-634.
- [33] M. Buck, H. Schwalbe and C.M. Dobson, Characterization of conformational preferences in a partly folded protein by heteronuclear NMR spectroscopy: Assignment and secondary structure analysis of hen egg-white lysozyme in trifluoroethanol, *Biochemistry* 34 (1995) 13219-13232.
- [34] M. Buck, S.E. Radford and C.M. Dobson, A partially folded state of hen egg white lysozyme in trifluoroethanol: structural characterization and implications for protein folding, *Biochemistry* 32 (1993) 669-678.
- [35] A.M. Buswell and A.P.J. Middelberg, Critical Analysis of Lysozyme Refolding Kinetics, *Biotechnol. Prog.* 18 (2002) 470-475.
- [36] P. Roux, M. Delepierre, M.E. Goldberg and A.-F. Chaffotte, Kinetics of Secondary Structure Recovery during the Refolding of Reduced Hen Egg White Lysozyme, *J. Biol. Chem.* 272 (1997) 24843-24849.
- [37] B. Raman, T. Ramakrishna and C.M. Rao, Refolding of Denatured and Denatured/Reduced Lysozyme at High Concentrations, *J. Biol. Chem.* 271 (1996) 17067-17072.

Table I: Stopped-flow kinetic parameters from ECD and fluorescence for reduced HEWL mixing with SDS and DTAC

	stopped-flow ECD		stopped-flow fluorescence	
	k_1 / s^{-1}	k_2 / s^{-1}	k_1 / s^{-1}	k_2 / s^{-1}
5 mM SDS	232 \pm 7	12.3 \pm 1.4	230 \pm 14	-
10 mM SDS	271 \pm 5	8.7 \pm 1.7	169 \pm 8	1.8 \pm 0.6
50 mM SDS	334 \pm 18	10.2 \pm 1.9	130 \pm 5	0.7 \pm 0.3
10 mM DTAC	209 \pm 6	10.6 \pm 1.4	194 \pm 16	0.6 \pm 0.3 ^a
25mM DTAC	251 \pm 6	11.5 \pm 0.8	199 \pm 21	8.0 \pm 1.3
35 mM DTAC	260. \pm 6	12.3 \pm 2.5	202 \pm 24	7.6 \pm 0.6
oxidized HEWL : SDS	-	9.6	128	8.2

^a 10 mM is below the CMC of DTAC

Captions for figures and tables

Fig. 1. (a) Far-UV CD spectra of native HEWL and reduced HEWL, and with addition of different concentrations of SDS at pH 4.6. (b) Calculated secondary structure fractions: helix (pink circles), sheet (green triangles) and unordered structures (blue squares).

Fig. 2. (a) Fluorescence spectra of native HEWL and reduced HEWL, and with addition of different concentrations of SDS at pH 4.6. (b) Variation of the integrated fluorescence intensity (squares) and peak position (triangles) with addition of anionic surfactant SDS (red circles show the peak positions of oxidized HEWL and oxidized HEWL in 10 mM SDS).

Fig. 3. Comparison of surfactant effects on native HEWL, ECD (left) and fluorescence (right) spectra of native HEWL and HEWL with addition of SDS and DTAC above their CMC.

Fig. 4. Near-UV CD spectra of native HEWL (red), reduced HEWL (green), native HEWL in 50 mM SDS (blue) and reduced HEWL in 50 mM SDS (pink). [Note these CD, as measured, are very weak due to a need to use shorter paths for the reduced samples.]

Fig. 5. (a) Variation of fractional secondary structure for reduced HEWL on addition of cationic surfactant DTAC, from fitting CD: helical (pink circles), sheet (green triangles) and unordered structures (blue squares). (b) Similar variation of fluorescence integrated intensity (blue squares) and peak position (black triangles).

Fig. 6. Thermal denaturation of native HEWL in buffer (filled squares), and with DTAC (diamonds) and SDS (unfilled squares), and reduced HEWL in buffer (unfilled triangles), and with DTAC (crosses) and SDS (filled triangles)

Fig. 7. Kinetic variation of reduced HEWL spectral response on mixing with SDS. Fitted curves for (a) the CD signal at 222 nm (an example of actual CD kinetic data is in Fig. S8, Supplement) and (b) the fluorescence intensity, where inset shows early time behavior.

Fig. 8. Kinetic variation of reduced HEWL spectral response on mixing with DTAC. Fitted curves for (a) the CD signal at 222 nm and (b) the fluorescence intensity, where inset shows early time behavior.

Scheme 1. Proposed mechanism for refolding of reduced HEWL, showing the possibility of multiple intermediates.

Table I: Stopped-flow kinetic parameters from ECD and fluorescence for reduced HEWL mixing with SDS and DTAC

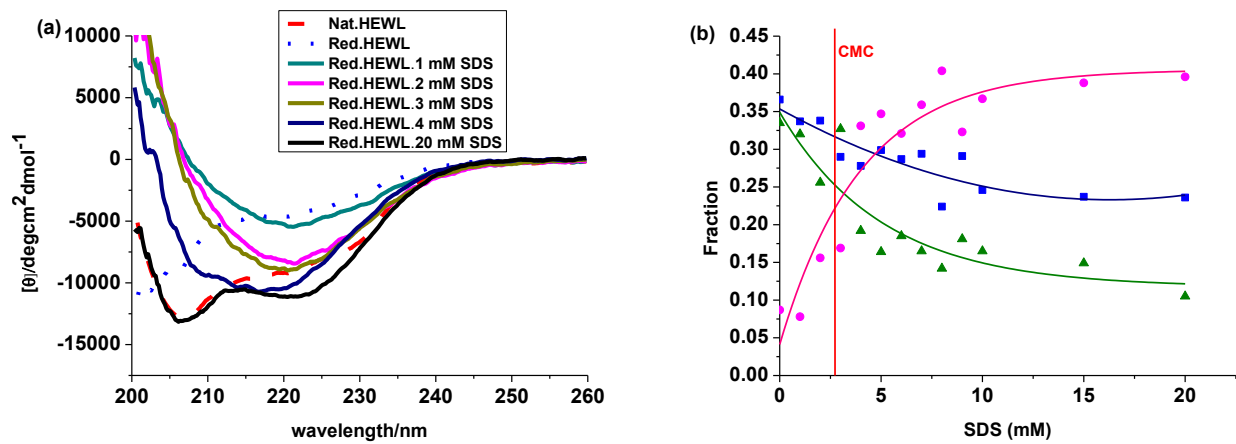


Fig 1. (a) Far-UV CD spectra of native HEWL and reduced HEWL, and with addition of different concentrations of SDS at pH 4.6. (b) Calculated secondary structure fractions: helix (pink circles), sheet (green triangles) and unordered structures (blue squares).

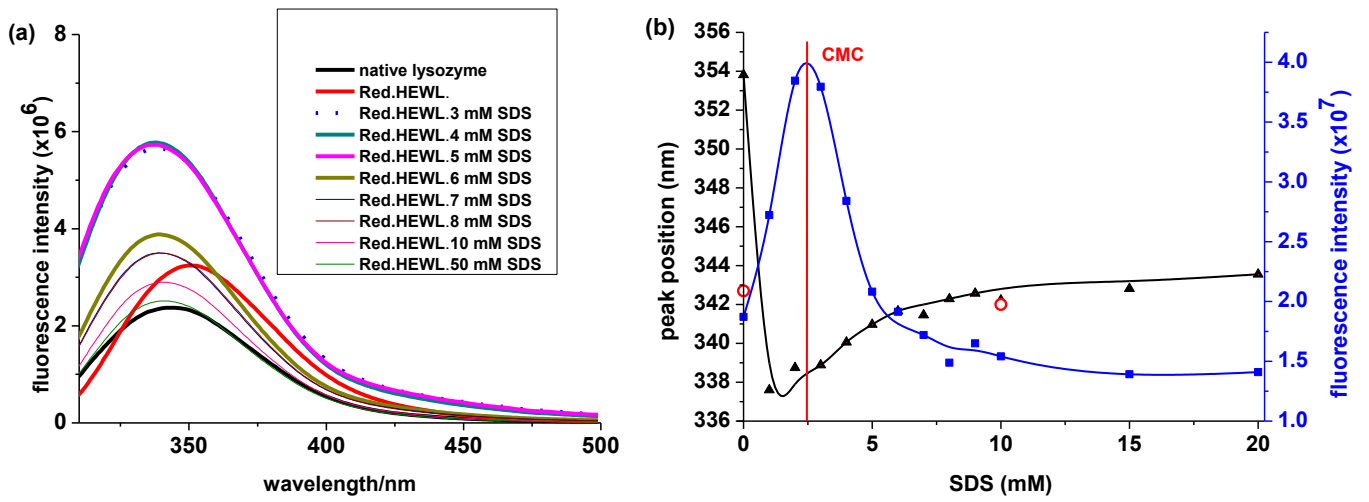


Fig 2. (a) Fluorescence spectra of native HEWL and reduced HEWL, and with addition of different concentrations of SDS at pH 4.6. (b) Variation of the integrated fluorescence intensity (squares) and peak position (triangles) with addition of anionic surfactant SDS (red circles show the peak positions of oxidized HEWL and oxidized HEWL in 10 mM SDS).

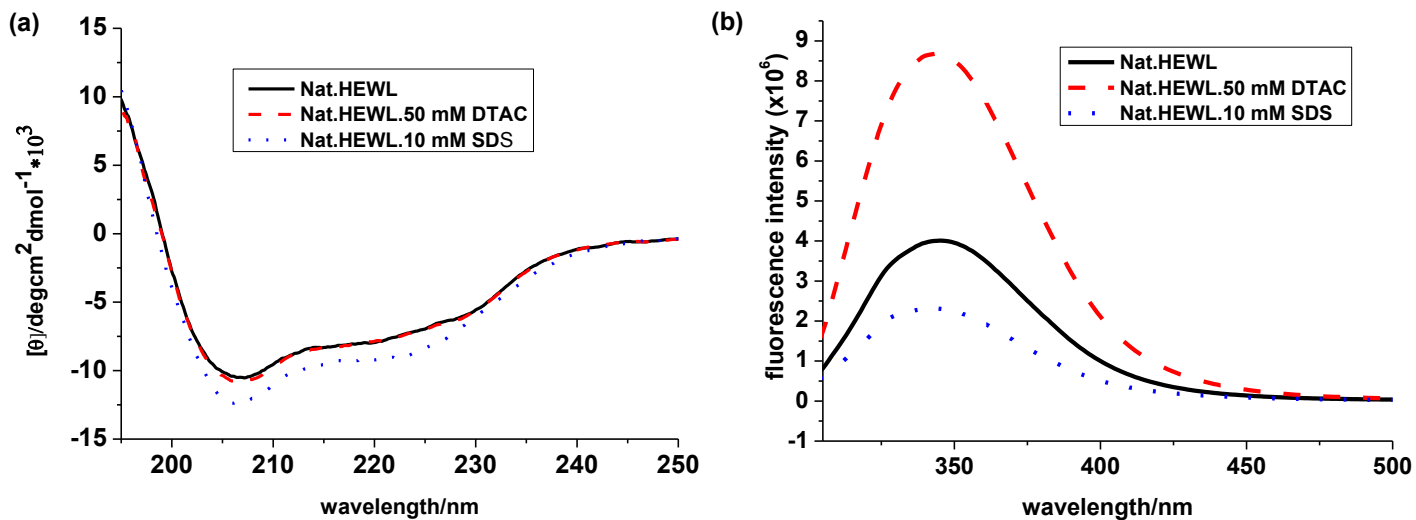


Fig. 3. Comparison of surfactant effects on native HEWL, ECD (left) and fluorescence (right) spectra of native HEWL and HEWL with addition of SDS and DTAC above their CMC.

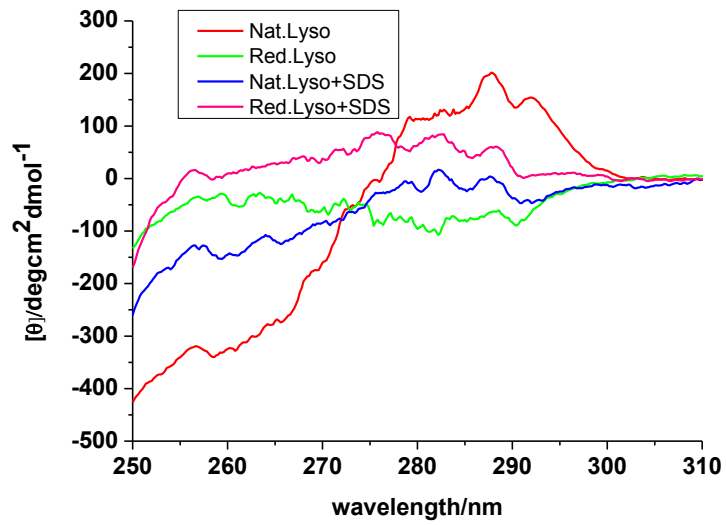


Fig. 4. Near-UV CD spectra of native HEWL (red), reduced HEWL (green), native HEWL in 50 mM SDS (blue) and reduced HEWL in 50 mM SDS (pink). [Note these CD, as measured, are very weak due to a need to use shorter paths for the reduced samples.]

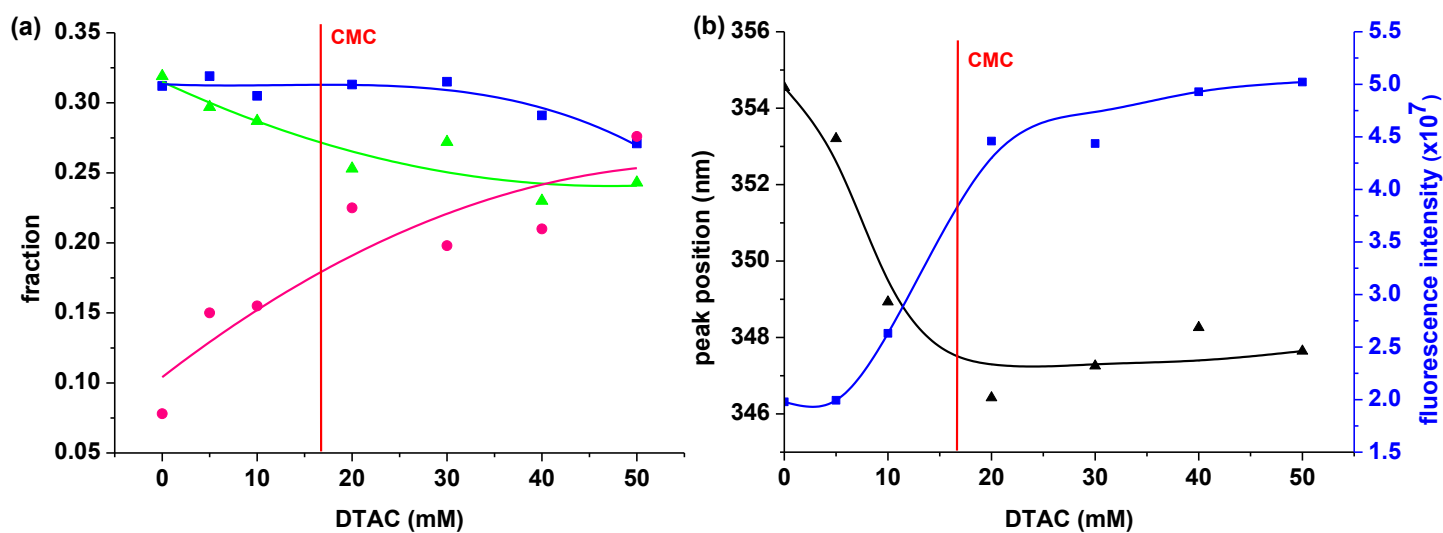


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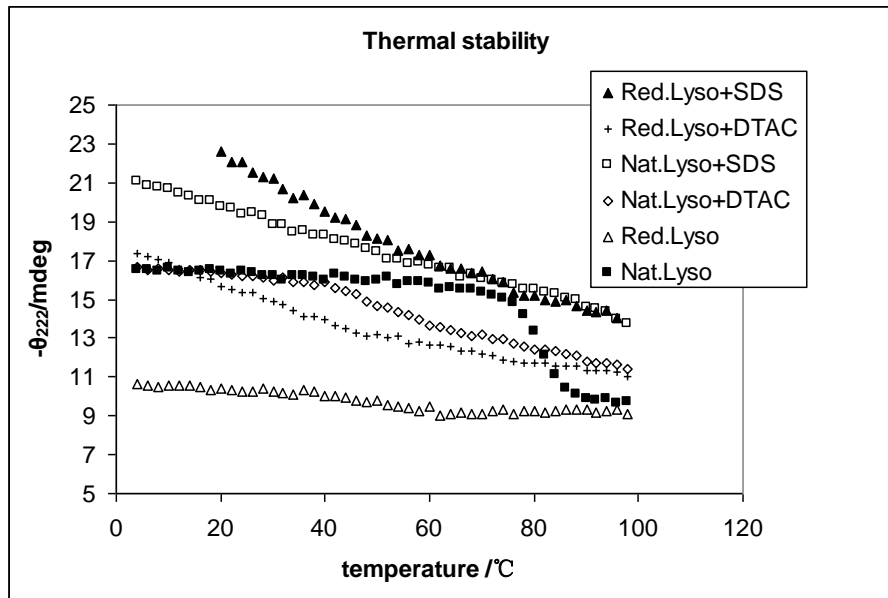


Fig. 6. Thermal denaturation of native HEWL in buffer (filled squares), and with DTAC (diamonds) and SDS (unfilled squares), and reduced HEWL in buffer (unfilled triangles), and with DTAC (crosses) and SDS (filled triangles)

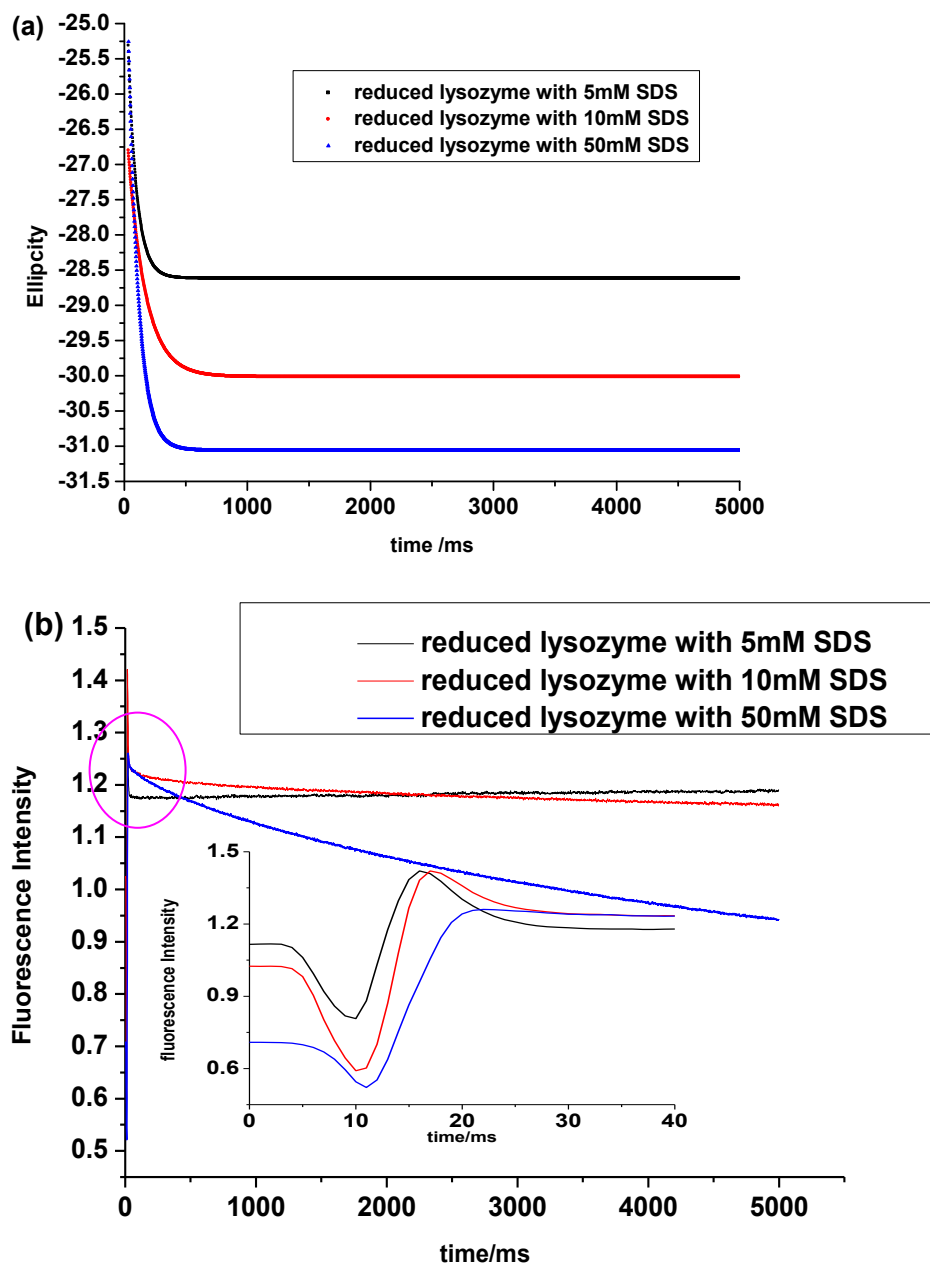


Fig. 7. Kinetic variation of reduced HEWL spectral response on mixing with SDS. (a) Fitted curves for the CD signal at 222 nm (an example of actual CD kinetic data is in Fig. S8, Supplement) and (b) Curves for the fluorescence intensity, where inset shows early time behavior.

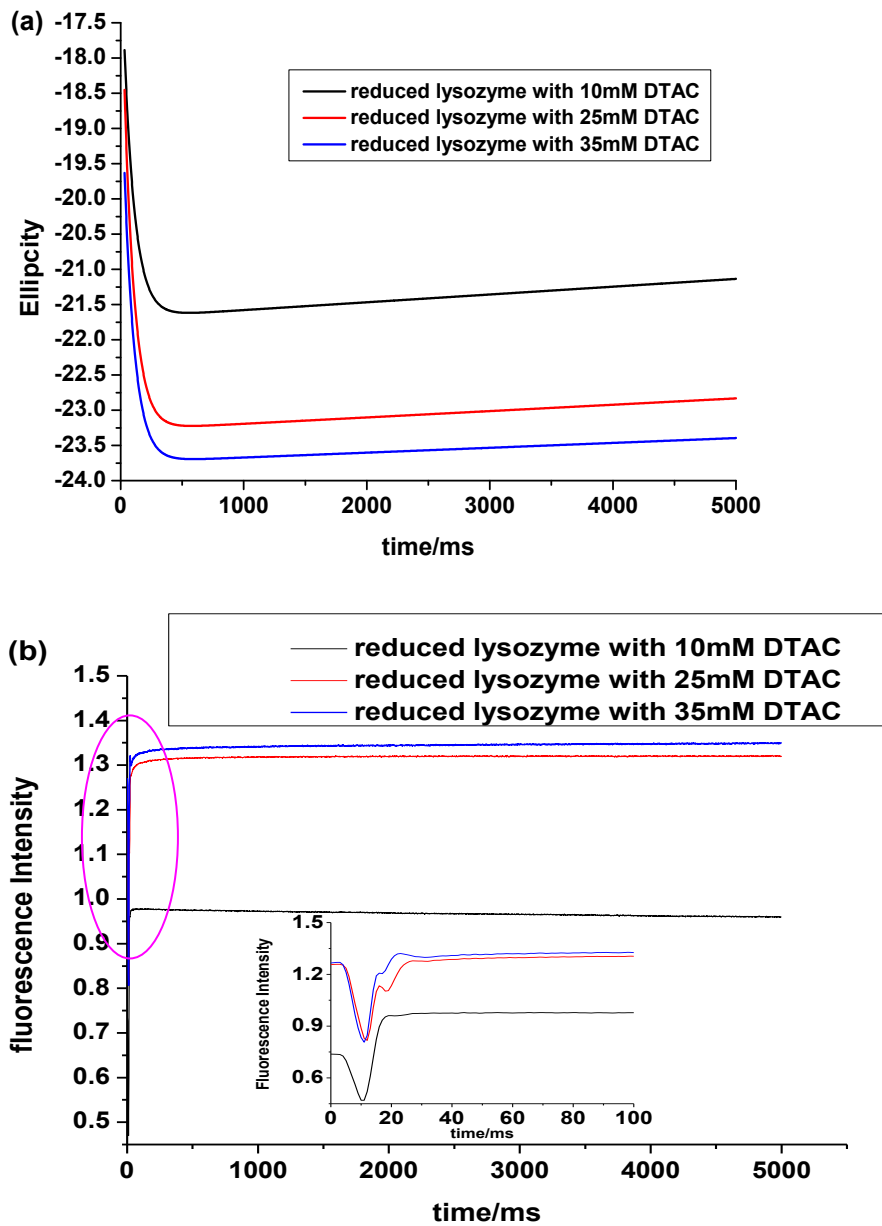
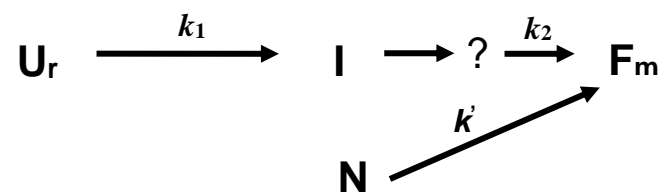


Fig. 8. Kinetic variation of reduced HEWL spectral response on mixing with DTAC. Fitted curves for (a) the CD signal at 222 nm and (b) the fluorescence intensity, where inset shows early time behavior.



Scheme 1. Proposed mechanism for refolding of reduced HEWL, showing the possibility of multiple intermediates.